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GEORGE T. LEWIS

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
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THE METABOLISM OF SULFUR.

XII. THE VALUE OF DIGLYCYL-CYSTINE, DIALANYL-CYSTINE, AND DIALANYL-CYSTINE DIANHYDRIDE FOR THE NUTRI- TIVE REQUIREMENTS OF THE WHITE RAT.

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(Received for publication, March 18, 1927.)

Studies of the physiological behavior of the peptides and their derivatives are not of frequent occurrence in the literature of biochemistry. Particularly is this true of the peptides, in whose molecule the amino acid cystine is contained. Interest in these compounds has been greatly stimulated by the discovery of glutathione (1), a peptide of cystine or cysteine and glutamic acid, as well as by the recent studies of the sulfur content of insulin (2) and of the change in the stability of the cystine sulfur in peptides and piperazines containing this amino acid by Bergmann and Stather (3) and by Brand and Sandberg (4).

In this laboratory we have been interested in the physiological behavior of organic compounds containing sulfur, particularly derivatives of cystine (5-8), and have been able to demonstrate that by protecting the amino group of cystine so that deamination is prevented, the oxidation of the sulfur fraction of cystine does not follow its normal course (5, 7). We have also observed that, although taurine is probably derived from cystine, taurine cannot replace cystine for purposes of growth in the young white rat (8), even under conditions of an urgent demand for cystine, a conclusion which was reached also by Rose and Huddlestun (9). In the present study, we have investigated the utilization for purposes of growth by the young white rat of two cystine-containing peptides, diglycyl-cystine and dialanyl-cystine, and of the 2, 5-diketopiperazine derived from dialanyl-cystine, dialanyl-cystine dianhydride. The behavior of this last compound has

seemed of unusual interest in view of the possible occurrence of piperazine rings in the protein molecule and of the marked lability of the cystine sulfur in this particular anhydride (3, 4).

Pepsin failed to split any of the synthetic peptides prepared by Fischer. Included in the list of those studied (10) were dialanyl-cystine and dileucyl-cystine, which were, however, readily hydrolyzed by the pancreatic juice of the dog. Glutathione (glutaminy-l-cysteine), on the other hand, is probably able to function in the organism because of its resistance to hydrolysis by enzymes and to other like metabolic changes (1). The action of the enzymes of the alimentary tract on diglycyl-cystine has not been investigated. Abderhalden and Samuely (11) have compared the absorption and oxidation of cystine and cystine in combination as a dipeptide, dialanyl-cystine or dileucyl-cystine, in experiments on dogs. If cystine was fed by mouth, 80 to 95 per cent of the administered sulfur was eliminated within 48 hours by the kidneys, for the most part as oxidized sulfur. When an equivalent amount of cystine was fed as dialanyl-cystine, 70 per cent of the ingested sulfur appeared in the urine within the same period of time. With dileucyl-cystine, the recovery of sulfur in the urine was slightly lower (64 per cent). When the dipeptides were injected subcutaneously as the sodium salts, the percentages of injected sulfur appearing in the urine within 48 hours were approximately the same (73.3 and 63.7 per cent for dialanyl-cystine and 63.8 per cent for dileucyl-cystine). In the dog, dialanyl-cystine was absorbed, either before or after hydrolysis, and the sulfur was metabolized to nearly the same extent as when pure cystine was fed. Even though dialanyl-cystine is not readily soluble, absorption was not at fault, since the same recovery of extra sulfur was obtained after subcutaneous injection as after oral administration.

The fate of the anhydrides of peptides (2, 5-diketopiperazines) in the animal organism has been studied by Abderhalden and collaborators (12-14). The behavior of these compounds, when fed by mouth, appears to vary with the nature of the amino acids present and to some extent with the individual animal. In some cases, the anhydride was excreted unchanged in the urine for the most part; in others, it was hydrolyzed and the resultant amino acids or peptides, either excreted by the kidneys, metabolized, or stored. In all experiments, the compounds appeared to

be readily absorbed from the alimentary canal, despite their marked insolubility in water. Brand and Sandberg (4) injected intravenously and intraperitoneally the anhydride of dialanyl-cystine into fasting rabbits and observed no toxic effects nor influence on blood sugar concentration. The amounts injected were, however, very small (about 13 and 60 mg. per kilo).

Our experiments have given evidence that diglycyl-cystine and dialanyl-cystine are as well utilized by the white rat for growth as an equivalent amount of cystine. When the anhydride of dialanyl-cystine was fed, however, no increased rate of growth as compared with that of the rats on the basal cystine-deficient diet was noted.

TABLE I.
Composition of Diets.

	Basal.	Diet G.	Diet H.	Diet I.	Diet J.
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
Casein.....	9.0	9.0	9.0	9.0	9.0
Salt mixture.....	4.5	4.5	4.5	4.5	4.5
Corn-starch.....	54.5	54.5	54.5	54.5	54.5
Sucrose.....	4.5	4.5	4.5	4.5	4.5
Lard.....	24.5	24.5	24.5	24.5	24.5
Cod liver oil.....	3.0	3.0	3.0	3.0	3.0
Cystine.....		0.34			
Diglycyl-cystine.....			0.50		
Dialanyl-cystine.....				0.54	
“ dianhydride.....					0.49

EXPERIMENTAL.

The diglycyl-cystine used in the experiments was prepared in this laboratory¹ by the method of Abderhalden and Spinner (15). Dr. Erwin Brand of the Montefiore Hospital, New York City, very kindly placed at our disposal the dialanyl-cystine and its dianhydride. The methods used in their preparation have already been described by him (4).

The general procedure of our animal experiments was the same as that previously reported (8). Young white rats of 30 to 50 gm. in weight were placed upon the basal cystine-deficient diet, the

¹ We are indebted to R. H. Wilson and F. H. Wiley for the preparation of the diglycyl-cystine.

basal diet plus cystine, and the basal diet plus the peptide or anhydride to be studied. In every case litter units were used, the litter mates serving as controls on cystine-deficient and cystine-containing diets. The composition of the diets is given in Table I. The amounts of the peptides, anhydride, and cystine were so adjusted that the diets contained equivalent amounts of cystine. The casein was a commercial product (Harris casein, free from vitamin B); the salt mixture was that of Osborne and Mendel. Water-soluble vitamin was supplied by Vegex, a commercial yeast concentrate.

DISCUSSION.

The results of the experiments, which comprised a series of five litters of rats, are presented in Table II.

It will be noted that the animals of Litters 13 and 21, which received the diglycyl-cystine, showed almost exactly the same increase in weight as did their litter mates whose diet contained an equivalent amount of cystine in place of the peptide. The rats of Litter 15, which were fed the peptide, did not make quite so great a gain in weight during the experimental period as did the animal which was fed cystine. In view of the results with the other litters, we do not feel that this difference is significant, especially since there is a very definite increase in the rate of growth over that of the animals of this litter, which received the basic cystine-deficient diet.

The results with dialanyl-cystine, although few in number, were equally definite and uniform. The two animals to which this compound was fed (Rats 191 and 195) during a period of 3 weeks showed very nearly the same increase in weight as did their litter mates maintained during the same period on the basal diet with the addition of an amount of cystine equivalent to the cystine content of the peptide.

Our experiments with dialanyl-cystine dianhydride were not quite so definite. Due to the small amount of the dianhydride available for our experiments we were not able to continue our feeding studies longer than 2 weeks with one rat (No. 192) and 3 weeks with another (No. 194). Rat 192 did not eat well while on the diet containing the dianhydride, its average daily food consumption for the 2 weeks being 4.9 and 4.5 gm. only, as compared

with an average daily intake of approximately 6 gm. of food for the control rats of the same age and weight. It may be noted, however, that Rat 126, during the 2nd week of the experimental

TABLE II.

Litter No.	Rat No.	Dura- tion.	Diet.	Initial weight.	Final weight.	Gain.
		<i>wks.</i>		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
13	113	5	Basal and diglycyl-cystine.	55.5	129.5	74.0
	114	5	" " "	55.0	122.0	67.0
	115	5	Basal.	56.0	100.5	44.5
	116	5	"	61.0	100.5	39.5
	117	5	" and cystine.	50.5	123.0	72.5
15	124	5½	Basal and diglycyl-cystine.	50.5	111.0	60.5
	125	5½	" " "	51.0	115.0	64.0
	126	5½	Basal.	54.0	94.0	40.0
	127	5½	"	55.0	97.5	42.5
	128	5½	" and cystine.	55.0	139.5	84.5
21	179	6	Basal.	44.5	94.5	50.0
	180	6	"	41.5	93.0	51.5
	181	7	" and diglycyl-cystine.	43.5	116.0	72.5
	182	6	" " "	44.0	128.5	84.5
	183	6	Basal and cystine.	39.0	121.0	82.0
	184	6	" " "	37.5	128.0	90.5
22	187	5	Basal.	54.5	96.5	42.0
	188	3	"	58.0	77.5	19.5
	189	5	" and cystine.	55.0	140.5	85.5
	190	3	" " "	58.5	107.5	49.0
	191	3	" " dialanyl-cystine.	45.0	93.0	48.0
	192	2	" " anhydride.	47.0	47.0	0.0
		1	" " dialanyl-cystine.	47.0	67.5	20.5
23	193	4	Basal and cystine.	50.5	110.0	59.5
		3	" " anhydride.	49.5	65.0	15.5
	194	1	" " dialanyl-cystine.	65.0	88.5	23.5
	195	3	" " "	48.0	92.5	44.5
	197	4	"	48.5	84.0	35.5

period, had an average daily food intake of only 4.9 gm., and was able, nevertheless, to maintain its body weight. Furthermore, this animal weighed 66 gm. while Rat 192 during the period under discussion weighed about 47 gm. and its food requirements for

maintenance must have been considerably less than those of Rat 126. Similarly, Rat 124, with an average daily food intake of 5.0 and 4.7 gm. during 2 successive weeks, gained 5 and 4.5 gm. respectively. Numerous other instances of the same kind might be cited from the records of the food intake, and hence it seems hardly probable that failure to grow was due entirely to the small amount of food eaten.

In the case of Rat 194, the food intake was greater and the animal showed a slow gain in weight while fed the diet containing the dianhydride. This increase, however, was only a little more than half that made by the control littermate on the 9 per cent casein diet without added cystine. During the last week of the 3 week period of the experiment, the two animals gained exactly the same amount, 10.5 gm. It seems reasonable to suppose that, if it had been possible to continue the experiment over a longer period of time, the rat on the diet containing the dianhydride would have shown a rate of growth similar to the control on the 9 per cent casein diet.

Both Rat 192 and Rat 194 were maintained for 1 week on a diet containing dialanyl-cystine after the feeding of the dianhydride was discontinued. In each case, a markedly increased rate of growth resulted. Rat 192 gained 20.5 gm. in 1 week and continued to gain at this rapid rate in the 2 following weeks on a cystine diet, so that its failure to show satisfactory growth on the anhydride diet was not due to lack of ability to grow when given an adequate diet. Similarly Rat 194, which gained 10.5 gm. during the 3rd week on a diet containing the dianhydride, made a gain of more than double this amount (23.5 gm.) in a week, when placed on a diet containing the dialanyl-cystine.

Among the possible causes of the failure of the dialanyl-cystine dianhydride to serve as an available source of cystine, the question of its absorption from the alimentary canal must be considered. As previously noted, Abderhalden and his collaborators (12-14) found that the anhydrides, which they studied, although very sparingly soluble, were readily absorbed. We have made use of the marked lability of the sulfur in dialanyl-cystine dianhydride (3, 4) in an attempt to determine whether failure of absorption of this substance was responsible for the results we have obtained. The sulfur of this compound is readily split by dilute alkali, even in the cold, whereas the peptide is less readily attacked and the

sulfur of the cystine is rather stable under these conditions. If, then, the dianhydride was not absorbed, but was excreted in the feces, the feces of a rat on a diet containing the dianhydride should contain significant amounts of labile sulfur. Feces were collected for a period of a week from three rats, one fed cystine, one dial-anyl-cystine, and one the dianhydride in addition to the basal diet. 1 gm. of each of the three samples of feces was heated in a boiling water bath for 15 minutes with 10 cc. of 10 per cent sodium hydroxide. The tubes were cooled, neutralized, and acidified with hydrochloric acid. Strips of filter paper moistened with lead acetate solution were suspended in the mouth of the tubes, which were then tightly stoppered. On standing for a few minutes, hydrogen sulfide in appreciable amount was liberated but no differences could be observed in the evolution of hydrogen sulfide as evidenced by the blackening of the lead acetate paper.

Since it seemed possible that this experiment might not be sufficiently delicate, we have carried out a roughly quantitative experiment. 0.5 gm. portions of the same samples of feces were finely pulverized, placed in test-tubes with 10 cc. of *N* sodium hydroxide, and allowed to stand at room temperature in contact with the alkali for 30 minutes. According to Brand and Sandberg (4), the sulfur of the dianhydride is readily split off under these conditions, while the sulfur of the corresponding dipeptide and of cystine is not greatly affected. The contents of each were filtered off and to the clear but rather deeply colored filtrates 10 drops of saturated lead acetate were added. The tubes were stoppered and allowed to stand for 24 hours. On the addition of the lead acetate to the filtrate from the feces of the animal which was fed the dianhydride, the development of the blackening was more rapid than with the other filtrates, and a decided precipitate was noted. After 24 hours, all three tubes contained a precipitate. The precipitates were filtered off in weighed Gooch crucibles and dried to constant weight at 100°. The weights of the lead sulfide were 5.6 mg. (cystine diet), 10.0 mg. (anhydride diet), and 7.7 mg. (peptide diet). For the weekly period, this would represent an excretion through the feces of 3.0, 5.4., and 4.1 mg. of labile sulfur respectively. Inasmuch as the sulfur intake (excluding the sulfur of the casein and salt mixture) was about 35, 36, and 49 mg. of sulfur respectively during the same period, this represents an unimportant loss of sulfur. When one considers the greater degree

of lability of the sulfur of the dianhydride as compared with the peptide or cystine itself, it seems impossible that lack of absorption is responsible for our negative results with the dianhydride, and that some other explanation, possibly inability to cleave the piperazine linkage,² must be sought. We cannot, of course, exclude the possibility that the dianhydride may be changed in its passage through the alimentary canal so that its sulfur is in a less labile combination. This seems hardly probable, however.

SUMMARY.

Experiments are described in which diglycyl-cystine, dialanyl-cystine, and the dialanyl-cystine dianhydride were fed to growing white rats maintained on a diet deficient in its content of cystine. Evidence was obtained that diglycyl-cystine and dialanyl-cystine were able to promote growth in the absence of an adequate supply of cystine. There were no indications that the dianhydride of dialanyl-cystine could be utilized for growth under these conditions. It is not believed that the unsatisfactory growth on the dianhydride is to be ascribed to failure of absorption of this constituent of the diet from the alimentary canal.

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² It is generally accepted that the diketopiperazine ring is not split by pepsin or trypsin. Levene and Pfaltz (16) recently stated that erepsin is also unable to effect this hydrolysis.

THE METABOLISM OF SULFUR.

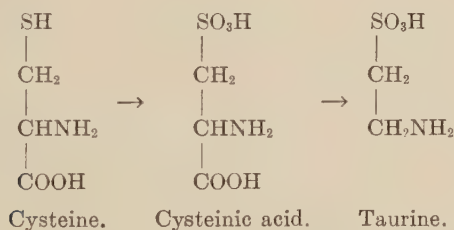
XI. CAN TAURINE REPLACE CYSTINE IN THE DIET OF THE YOUNG WHITE RAT?*

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(Received for publication, June 14, 1926.)

Of the organic sulfur compounds of biological interest, taurine is unique as the only sulfonic acid derivative known to have its origin in the activity of living cells. Its origin from the cystine of the protein molecule is generally accepted, but proof of the reaction by which it is derived is lacking. Friedmann (1) oxidized cystine to cysteinic acid by bromine water, and was successful in obtaining taurine from the latter by decarboxylation at high temperatures.¹



Evidence has been presented by von Bergmann (2), Wohlge-muth (3), and Foster, Hooper, and Whipple (4), which indicates that the taurocholic acid of the bile is dependent upon the animal's

* A preliminary report of part of this work appears in *Proc. Soc. Exp. Biol. and Med.*, 1926, xxiii, 359.

¹ Considerable difficulty has been experienced in frequent attempts to repeat Friedmann's conversion of cysteinic acid to taurine in this laboratory. Recently, however, Miss Genevieve Stearns has been able to obtain a satisfactory product which showed a high degree of purity on analysis.

supply of cystine as a source of the taurine for the conjugated bile acid. Gibson (5) attempted to deplete the available supply of cystine by the administration of bromobenzene to cats, in the expectation that the utilization of the cystine for the synthesis of the bromophenylmercapturic acid would diminish the amount available for the synthesis of taurine and thus lessen the output of alcohol-soluble sulfur (assumed to be present largely as taurocholic acid) of the bile. His work also indicates a relationship between cystine and taurine.

If the origin of taurine in the organism from cystine through cysteinic acid be accepted, the question of the reversibility of the reactions by which taurine is formed becomes of interest, particularly in view of the problem of amino acid synthesis in the animal organism. Can the reaction be reversed when the supply of taurine is abundant and the need for cystine is urgent?

In certain species, notably the dog, cystine is utilized for the detoxication of bromobenzene with the formation of bromophenylmercapturic acid. In fasting animals poisoned with bromobenzene, administration of cystine has been known to increase the production of the mercapturic acid (6). If to such animals, taurine is fed, the output of the mercapturic acid should be increased, if the formation of cystine from taurine is possible. In experiments designed to test out this possibility, Muldoon, Shipley, and Sherwin (7) were unable to obtain any evidence of the conversion of taurine to cystine.

Osborne and Mendel (8) and others have shown that with diets whose protein is derived chiefly from casein (9 per cent), cystine becomes the limiting factor for the growth of young white rats. Beard (9) has demonstrated that cystine is also the limiting amino acid factor in casein, when this protein is fed at a concentration of 12 per cent, in the diet of mice. If taurine can replace cystine, then white rats or mice fed on a basal diet of this type, deficient in cystine, should show equally great increments in growth when taurine supplements the basal diet as when cystine is added. Mitchell (10) has carried out experiments of this kind with mice. He employed a modification of the Osborne-Mendel diet and obtained results which indicated that cystine and taurine were equally efficient in producing an increased rate of growth over that obtained with the basal diet. In considering the results, Mitchell

points out that they are not necessarily attributable to a conversion of taurine to cystine, but merely indicate that the animal can utilize taurine for purposes of growth in the absence of sufficient cystine. Beard (9), however, was unable to confirm the results of Mitchell. It should be noted that the diets used by these two observers were not exactly comparable, since a higher level of casein (12 per cent) was furnished in the experiments of Beard. It is possible that with the lower level of casein (9 per cent) the need for cystine was more acute and its synthesis more easily facilitated in the experiments of Mitchell.

The experiments reported in the present communication were undertaken to determine whether taurine could replace cystine for purposes of growth in the diet of young white rats and also whether the product intermediary between taurine and cystine, cysteinic acid, could function in the nutrition of these animals in place of cystine. The results of our experiments with two types of deficient basal diets have been uniformly negative and have failed entirely to give any evidence that either taurine or cysteinic acid can replace cystine for purposes of *growth* in the young white rat.

EXPERIMENTAL.

Young white rats of about 30 to 50 gm. weight were used as the experimental animals. Each litter was divided into three groups. One group received the basal (cystine-deficient) diet; the second, the same basal diet with added cystine; and the third, the basal diet plus taurine or cysteinic acid.

Two types of low cystine basal diets were fed, the low protein diet of Osborne and Mendel in which casein is the sole source of protein, and the milk powder-corn-starch diet of Sherman and Merrill (11). The composition of the diets fed is given in Tables I and II. For a short period during the experiments, the modified diet of Sherman and Woods (12) was fed, but the animals did not eat this as readily and scattered more of the food in the cage, so that its use was discontinued. The casein (Harris) was free from vitamin B. The salt mixture of Osborne and Mendel was used. A commercial dry milk powder (Klim) was employed in the diets of the Sherman type. In earlier experiments, the water-soluble vitamin (50 mg. daily) was supplied by yeast concentrate tablets,

but in later work, Vegex was substituted for these. Each rat received approximately 75 mg. daily. The paste was placed in the food cup and was eaten readily. Cystine was prepared from hair, taurine from ox bile, and cysteinic acid by the oxidation of cystine (1).

TABLE I.
Composition of the Sherman Diets.

	Basal.	Diet A.	Diet B.	Diet C.
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
Corn-starch.....	81.8	81.8	81.8	81.8
Milk powder.....	16.6	16.6	16.6	16.6
Sodium chloride.....	1.6	1.6	1.6	1.6
Cystine.....		0.3		
Taurine.....			1.0	
Cysteinic acid.....				0.5

TABLE II.
Composition of the Osborne-Mendel Low Protein Diets.

	Basal.	Diet D.	Diet E.	Diet F.
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
Casein.....	9.0	9.0	9.0	9.0
Salt mixture.....	4.5	4.5	4.5	4.5
Corn-starch.....	54.5	54.5	54.5	54.5
Sucrose.....	4.5	4.5	4.5	4.5
Lard.....	24.5	24.5	24.5	24.5
Cod liver oil.....	3.0	3.0	3.0	3.0
Cystine.....		0.5		
Taurine.....			1.0	
Cysteinic acid.....				0.5

DISCUSSION.

The results of the series in which the milk powder-starch diet was fed are presented in Table III. It will be noted that in every case, the animals which received cystine showed a more rapid increase in weight than their litter mates on the basal diet, while when taurine or cysteinic acid (Litter 6) was added, no similar increased rate of growth was obtained. The increase in weight resulting from the addition of cystine to the Sherman diets seems to be somewhat variable although fairly constant for each litter.

TABLE III.
Summary of the Experiments with Sherman Diet.

	Dura- tion.	Diet.	No. of rats.	Initial weight.	Final weight.	Gain.
	<i>wks.</i>			<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
Litter 1, Rats 25 to 34.	10	Basal.	3	57.0	120.5	63.5
				55.0	109.5	54.5
				47.0	95.0	48.0
		Basal and taurine.	3	50.5	95.5	45.0
				50.5	91.0	40.5
				48.0	99.5	51.5
		Basal and cystine.	3	47.0	132.0	85.0
				47.5	141.5	94.0
				41.0	112.5	71.5
Litter 2, Rats 35 to 40.	10	Basal.	2	84.0	130.0	46.0
				85.0	125.5	40.5
		Basal and taurine.	2	84.5	129.5	45.0
				88.5	124.5	36.0
		Basal and cystine.	2	79.0	147.5	68.5
				54.0	128.0	74.0
Litter 3, Rats 49 to 54.	8	Basal.	2	27.5	72.0	44.5
				29.0	75.5	46.5
		Basal and taurine.	2	32.0	84.5	52.5
				31.5	70.0	38.5
		Basal and cystine.	2	26.0	80.0	54.0
				26.0	93.0	67.0
Litter 4, Rats 55 to 58.	10	Basal.	2	30.5	82.5	52.0
				30.5	82.0	51.5
		Basal and taurine.	1	29.0	94.5	65.5
		Basal and cystine.	1	29.0	120.0	91.0
		Basal.	2	41.5	83.5	42.0
				49.0	98.0	49.0
Litter 5, Rats 59 to 66.	9	Basal and taurine.	3	41.0	84.0	43.0
				39.0	85.0	46.0
				43.0	82.5	39.5
		Basal and cystine.	3	37.0	95.0	58.0
				38.5	93.5	55.0
				36.0	101.0	65.0
		Basal.	2	41.5	83.5	42.0
				49.0	98.0	49.0
				41.0	84.0	43.0

TABLE III—*Concluded.*

	Dura- tion.	Diet.	No. of rats.	Initial weight.	Final weight.	Gain.
	<i>wks.</i>			<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
Litter 6, Rats 67 to 75, 79.	8	Basal.	3	38.0	84.0	46.0
				37.0	81.5	44.5
				39.5	81.5	42.0
		Basal and cysteinic acid.	3	34.0	74.5	40.5
				35.5	76.5	41.0
				34.0	82.0	48.0
		Basal and cystine.	3	25.5	76.0	50.5*
				29.0	98.5	69.5
				38.5	93.5	55.0*

* Diarrhea.

Sherman (11) states that the previous diet of the parents influences the response of the young rats to added cystine. This may well be the explanation of our results, since our rats, particularly in the early experiments, were obtained from different breeders, and their

TABLE IV.

Average Increase in Weight in the Initial 6-Week Period of the Experimental Diets.

Litter No.	Basal.	Basal and taurine or cys- teinic acid.	Basal and cystine.	Gain in weight of cystine-fed animals as com- pared with control animals on basal diet.
	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
1	28.3	33.5	54.8	26.5
2	27.2	26.2	51.0	23.8
3	33.0	32.7	43.5	10.5
4	27.5	37.0*	48.0	20.5
5	26.2	26.8	35.7	9.5
6	34.2	30.7†	54.0	19.8

* See comment in text.

† Cysteinic acid.

parents may have had different dietary régimes. In the later experiments in which rats were bred in our own laboratory, the variability in response to added cystine between litters was less

marked. In only one animal (Litter 4) was there shown an appreciably greater gain in weight in the animal fed taurine as compared with the animals which received the basal diet. This increase in weight was, however, only about one-third that obtained with the cystine-fed animals, although the amount of taurine fed was more than three times the amount of cystine.

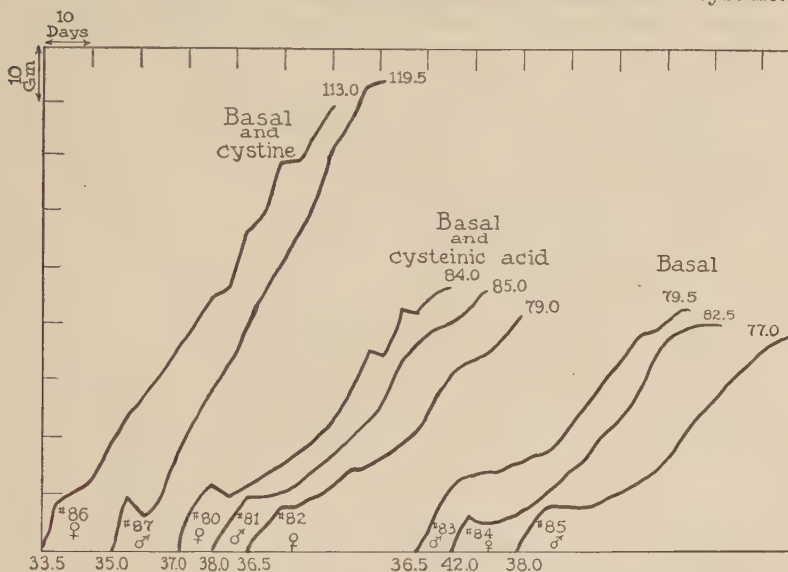


CHART I. Litter 7, showing the curves of growth of young white rats on diets low in cystine (basal diet), and on diets in which cystine and cysteinic acid supplemented the cystine-deficient diet. The addition of cystine (0.5 per cent) to the basal diet (Diet D) resulted in prompt response in growth, while growth on the diets in which cysteinic acid (0.5 per cent) supplemented the basal diet (Diet F) did not differ appreciably from that obtained on the basal diet.

It is not felt that the results with this one animal are significant in view of the results with other animals. The increased rate of growth can hardly be attributed to a greater food consumption by the cystine-fed animals. A record of the food intake was made in all experiments. The dry, loose character of the diet made it difficult to estimate the intake accurately, particularly with certain animals which were inclined to waste the food.

Sherman (11) states that the rats in his cystine series (0.2 per

cent) showed an average increase in weight of 28.6 gm. more than those on the basal diet during a 6 weeks period. The animals in our experiments which were fed diets containing slightly greater amounts (0.3 per cent) of added cystine, did not quite reach this figure in 6 weeks (Table IV). Two of the three rats in Litter 6 had a bad diarrhea, which probably accounts for their failure to show a greater gain in weight, although they did make slightly greater gains than their litter mates on the basal diet.

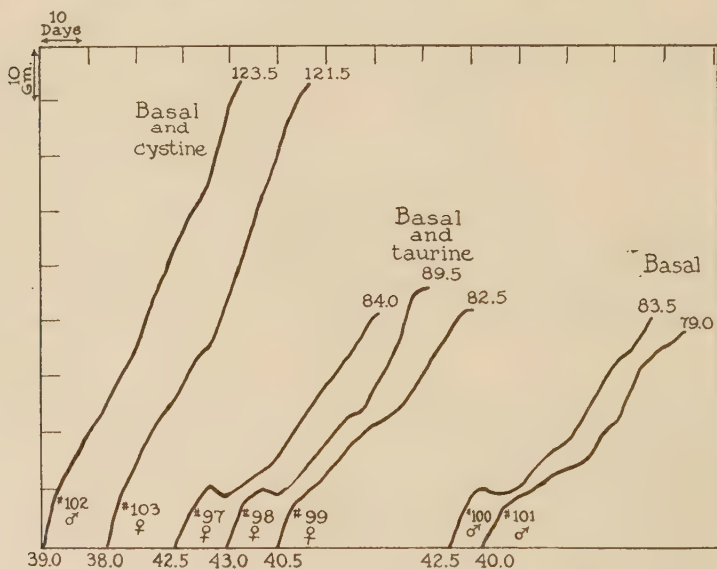


CHART II. Litter 8, showing the curves of growth of young white rats on diets low in cystine (basal diet), and on diets in which cystine and taurine supplemented the cystine-deficient diet. The addition of cystine (0.5 per cent) to the basal diet (Diet D) resulted in prompt response in growth, while growth on the diets in which taurine (1.0 per cent) supplemented the basal diet (Diet E) did not differ appreciably from that obtained on the basal diet.

Typical results obtained with rats on the Osborne-Mendel low protein diet are presented in Charts I and II and require little comment. They are in accord with the experiments just discussed in their failure to afford any evidence of the utilization of taurine or cysteic acid in place of cystine for purposes of growth.

It is difficult to correlate these results with those of Mitchell

(10). Since different species differ in the relative amounts of glycocholic and taurocholic acids present in the bile, it might be assumed that the need for taurine would vary in different species. If cystine is the precursor of taurine, it might be anticipated that in a species whose bile contained very considerable amounts of taurocholic acid, the demand for cystine would be greater than in a species in whose bile taurocholic acid is almost entirely lacking, and that in such an animal taurine might, in part at least, replace the cystine. We know of no analysis of the taurocholic and glycocholic acid contents of rat and mouse bile. If the bile of the mouse were shown to contain much taurocholic acid and that of the rat to contain little or no taurocholic acid, the discrepancy between our results with rats and those of Mitchell with mice might be explained. Beard (9), however, was unable to obtain results similar to those of Mitchell in his experiments with mice.

The taurine used in our experiments was obtained by hydrolysis of ox bile, while the taurine in Mitchell's experiment was isolated from the muscle of the abalone (*Haliotis*). Mitchell states that, if cystine were present in the preparation fed, it could have occurred only in traces. Professor C. L. A. Schmidt of the University of California kindly furnished us with a sample of taurine from abalone. It gave no color whatever in the Folin-Looney cystine reaction, which would appear to be more delicate than the Wollaston test employed by Mitchell. Neither did we obtain any different response to it when fed to a white rat over a period of 2 weeks than to the taurine prepared from bile. Mitchell does not state the percentage of taurine in his diets. Our diets contained as stated 1 per cent of taurine.

SUMMARY.

Experiments are described in which taurine and cysteinic acid were fed to young white rats on two types of diets, in which lack of cystine was the limiting factor. No evidence was obtained which indicated that either taurine or cysteinic acid could replace cystine entirely or in part for purposes of growth, in the absence of adequate amounts of cystine in the diet.

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THE METABOLISM OF SULFUR.

XIII. THE EFFECT OF ELEMENTARY SULFUR ON THE GROWTH OF THE YOUNG WHITE RAT.*

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(Received for publication, July 12, 1927.)

In this laboratory we have been interested in the physiological rôle of substances containing sulfur which might possibly be able to influence the requirements of cystine for purposes of growth in the young white rat. Thus we have been able to demonstrate that cystine combined as in a peptide (diglycyl- or dialanyl-cystine) was utilized effectively for growth (1), while neither the dianhydride of dialanyl-cystine (1), nor the oxidation products of cystine, cysteinic acid and taurine (2), could replace cystine for purposes of growth under the experimental conditions used by us. In continuation of this line of work, we have studied the effect on the rate of growth of the white rat of the addition of elementary sulfur (flowers of sulfur) to two types of diets in which cystine was the first limiting factor.

The addition of elementary sulfur to a diet deficient in cystine did not permit maintenance in adult white mice (3). In this series, in which only 3 animals were used and the experiments extended over very short periods (1 to 2 weeks), sulfur was added to the extent of 1 per cent of the diet. No toxic effects were recorded.

From the theoretical standpoint, any influence of elementary sulfur on the requirements for or utilization of cystine might be the result of either or both of two processes. Sulfur might under-

* The material presented in this paper and in Papers XI and XII of this series (1, 2) represents an abstract of a thesis presented by George T. Lewis in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the University of Michigan.

go transformation to some organic derivative and play a rôle in the actual synthesis of cystine in the body. Such a reaction would seem unlikely in view of the studies on the synthesis of various other essential amino-acids by the intact organism. More probable would be an effect of elementary sulfur on metabolism as a result of its conversion to sulfides, and the oxidation of the latter to sulfates by the body (4). This extra sulfate sulfur accumulated in the system might possibly act as a cystine sparer by depressing the rate of oxidation of the mercapto groups. Such an effect would have an analogy in the reported depression of the level of general protein catabolism by alteration of the rate of deamination if ammonium salts or urea are present in excess in the organism (5). Mass action of ingested ammonium salts or urea, products of deamination of the amino acids of the protein molecule, may prevent deamination of some amino acids and thus ammonium salts may function as protein spacers. Similarly sulfates, normal products of the oxidation of the sulfur of the cystine molecule, if present in amounts greater than normal, might depress the catabolic reactions in which cystine is concerned. On the basis of this theory, the addition of sulfates to a cystine-deficient diet might be expected to influence favorably the rate of growth. In the experiments of Daniels and Rich (6) however, the addition of inorganic sulfates to the basal diet did not alter the rate of growth of white rats maintained on a diet deficient in its content of cystine.

Our results have not only failed to demonstrate any favorable influence of sulfur when substituted for cystine at various levels in the diet of the white rat but have shown that continued oral administration of relatively small amounts of sulfur may produce slower rate of growth, definite toxic results, and in some cases, death of the experimental animals.

EXPERIMENTAL.

The experiments were carried out as described in our earlier publications (1, 2). Two types of basal cystine-deficient diets were used, the milk powder-starch diet of Sherman and Merrill (7) and the low protein (casein) diet of Osborne and Mendel (Table I). To these basal diets were added varying amounts of elementary sulfur (Baker's powdered flowers of sulfur), 0.08, 0.5,

0.75, and 1.0 per cent. Our earlier experiments were carried out with the higher percentages of sulfur and demonstrated, as already noted, a retardation of growth and marked toxicity.

In a series of controls, cystine (0.3 per cent) was supplied to demonstrate that addition of cystine to the unsatisfactory diets resulted in good growth. Since the amount of sulfur added to the diet in the earlier experiments far exceeded that added in the form of cystine in the control experiments, in a later series, an amount of sulfur equivalent to the sulfur content of cystine in the control cystine series (0.08 per cent sulfur) was added. It was felt that if sulfur could be utilized, this type of diet might be more favorable than those diets to which relatively large amounts

TABLE I.
Composition of Diets.

Sherman-Merrill diet.		Osborne-Mendel diet.	
	Basal.		Basal.
	gm.		gm.
Corn-starch.....	81.8	Casein.....	9.0
Milk powder.....	16.6	Salt mixture.....	4.5
Sodium chloride.....	1.6	Corn-starch.....	54.5
		Sucrose.....	4.5
		Lard.....	24.5
		Cod liver oil.....	3.0

Sulfur and cystine were added to these basal diets as indicated in the text.

of sulfur (viewed from the standpoint of their equivalents in cystine) were added.

A considerable number of the rats were killed after periods of 9 to 14 weeks and the glycogen contents of the livers were determined according to the method of Pflüger. After hydrolysis of the glycogen, the glucose was estimated colorimetrically (Folin-Wu). The livers, kidneys, and in a few cases, the spleens of a number of sulfur rats, as well as those of the controls, were submitted to the Department of Pathology for examination. We are indebted to Professors A. S. Warthin and Carl V. Weller for the pathological diagnoses.

DISCUSSION.

The series of rats fed the Sherman-Merrill diet included 10 litter units, a total of 73 animals. In order to condense the data, details concerning one litter only are presented in the tables. The most noticeable effect of the sulfur was that, during the first 2 or 3 weeks of the sulfur feeding, growth was much retarded or ceased altogether. Although this may have been due in part to diminished food consumption, this does not explain entirely the failure to grow. Usually the rats lost weight during the 1st week and then gained slowly, so that by the end of the 2nd week they had regained their initial weight. From this time, the rate of growth was nearly as rapid as that of the control rats on the cystine-deficient diet, but the total gain over a period of 7 to 9 weeks was not as great, since the rats fed sulfur never overcame the initial handicap of the first 2 weeks. The results suggest that the animals may have gradually acquired a tolerance for sulfur and that growth was possible only when such a tolerance was established.

Sulfur in as low a concentration as 0.5 per cent noticeably retarded the rate of growth. When the amount of sulfur added to the basal diet was increased to 0.75 per cent, retardation of growth was more marked. Thus in Litter 18, three control rats gained 60.5, 65.5, and 68.0 gm. respectively in 74 days as compared with gains of 45.0 and 47.0 gm. for their litter mates which received the basal diet with the addition of 0.75 per cent sulfur.

When the sulfur content of the diet was increased to 1 per cent, toxicity was more marked and of twenty-two animals, fourteen died in periods of from 3 to 21 days. None of the control animals in our entire series died during the course of the experiments. If an animal was able to overcome the toxic effect of the sulfur for the first 2 or 3 weeks, a tolerance for the sulfur was apparently developed and growth then occurred although at a slightly lower rate than in the control animals. It should be noted also that of the animals receiving small amounts of sulfur in the diet, one on a level of 0.5 per cent sulfur died in 29 days and two on the 0.75 per cent level died in 11 and 27 days respectively.

Since the basal diets were deficient in cystine, it seemed possible that the toxic action of the sulfur might be increased by the lack

of cystine and the consequent poor state of nutrition. We have accordingly supplemented the basal diet with 0.3 per cent of cystine and at the same time added 1.0 per cent of sulfur. This litter appeared to be especially susceptible to the toxic effect of the sulfur. All the rats which received sulfur died in 3 days; those which received cystine in addition survived no longer than their litter mates to whose diet cystine was not added.

Pathological examination of the liver and kidneys showed that the liver was the organ mainly affected. With a few exceptions, the livers of the rats which died showed a marked peripheral zonal necrosis. When the sulfur was not fed in sufficiently large

TABLE II.
Litter 26. Sherman-Merrill Diet.

Period, 6 weeks.

Rat No.	Diet.	Initial weight.	Final weight.	Gain.
		<i>gm.</i>	<i>gm.</i>	<i>gm.</i>
226	Basal.	43.5	73.5	30.0
227	"	40.5	61.0	20.5
228	"	39.0	70.0	31.0
229	" and sulfur 0.08 per cent.	44.0	72.0	28.0
230	" " " " " "	41.0	59.5	18.5
231	" " " " " "	38.5	66.0	27.5
232	" " " " " "	39.0	53.0	14.0
233	" " cystine 0.3 " "	32.0	77.5	45.5
234	" " " " " "	33.5	83.5	50.0
235	" " " " " "	35.0	82.5	47.5

amounts to cause death, the livers showed some pathological changes but not the typical zonal necrosis. The kidneys did not exhibit any constant pathological condition.

The results with Litter 26, which received an amount of sulfur (0.08 per cent) equivalent to the cystine which effectively supplements the defective basal diet, are presented in Table II. No marked toxicity was observed and with the exception of one animal (Rat 232) the rats receiving the sulfur increased in weight nearly as rapidly as did the control animals. Addition of cystine to the basal diet resulted in rapid gain.

In the experiments with the Osborne-Mendel diet (four litters,

twenty-two rats) only the highest concentration of sulfur (1 per cent) was used. Table III presents a summary of the results with a typical litter. The retardation of growth was similar to that observed with the Sherman-Merrill diet but none of the animals died. Despite a satisfactory food intake, as shown in the table, the rats receiving sulfur failed to grow normally and the gain per 100 gm. of food was decidedly less than the controls. Although rats receiving both cystine and sulfur (Rats 215, 216) gained slightly more rapidly than the controls with neither added cystine nor sulfur, their rate of growth was much less than was

TABLE III.
Osborne-Mendel Diet.

Period, 32 days.

Rat No.	Diet.	Initial weight.	Final weight.	Gain.	Total food.	Food per 100 gm.	Gain per 100 gm.
		gm.	gm.			rat.	food.
210	9% casein.	42.0	67.0	25.0	191.0	36.0	13.1
211	Same + 0.34% cystine.	35.0	85.0	50.0	191.5	32.1	26.0
212	" + 0.34% "	32.0	72.0	40.0	158.5	32.7	25.2
213	" + 1% sulfur.	52.0	65.5	13.5	149.0	29.5	9.1
214	" + 1% "	45.0	56.5	11.5	179.5	40.7	6.4
215	" + 0.34% cystine, 1% sulfur.	42.5	74.5	32.0	161.5	29.5	19.8
216	" + 0.34% " 1% "	42.5	67.5	25.0	147.5	30.6	16.9

to be expected in the presence of an adequate amount of cystine (*cf.* Rats 211, 212).

It remains to consider what was the immediate cause of death of the animals which died as a result of the administration of sulfur. While we have no direct evidence on this point, it seems probable that death was due to poisoning by hydrogen sulfide. Haibe (8) found that under conditions of chronic hydrogen sulfide poisoning, which is the condition with which we are concerned here, if the gas is being continually formed from sulfur in the intestine, the liver is the main organ affected. In many cases, we found hydrogen sulfide present in the abdominal cavity of rats to which sulfur had been fed. After the animal had died

from natural causes or had been killed with chloroform, the abdominal wall was exposed, a small incision made, and a strip of filter paper moistened with lead acetate was laid over the opening. In some cases, enough hydrogen sulfide was present to turn the paper a deep black, while in others, only a trace was detected. This was not a postmortem product because the number of positive tests was about equally divided between those animals examined some hours after death and those killed with chloroform and examined immediately. A darkening of the lead acetate was never obtained with the control rats which had never received sulfur in the diet.

The theory that the toxicity of the sulfur is due to the formation of hydrogen sulfide is confirmed by a consideration of the vast amount of pharmacological experimental data, which have been collected in connection with the therapeutic use of sulfur in various forms. Most investigators seem to consider that the hydrogen sulfide is a product of the action of intestinal bacteria (9). However, as shown by Heffter (10, 11) in the absence of bacteria, some proteins, especially egg white, and many animal tissues, including the intestinal mucosa, could form hydrogen sulfide from elementary sulfur. The formation of hydrogen sulfide in the intestine may be due to either or both reactions. Hydrogen sulfide is then absorbed, exerts its toxic action or is oxidized to sulfates (4).

It is difficult to explain the difference in toxicity of sulfur added to the Sherman-Merrill milk powder-starch diet and the Osborne-Mendel low protein diet. If the toxicity of the sulfur is due, as seems probable, to chronic hydrogen sulfide poisoning as a result of the formation of hydrogen sulfide by bacteria or by reaction with the intestinal mucosa, the lessened toxicity on the Osborne-Mendel diet might be explained by the higher fat content and slightly higher protein level. It seems possible that the fat might form a coating around the particles of sulfur and thus make more difficult the intimate contact of the sulfur with the intestinal mucosa or the action of bacteria. The higher protein level might also result in a better state of nutrition and hence a greater resistance to the toxic action. No simple explanation is evident for the apparent development of tolerance to the sulfur in the animals on the Sherman-Merrill diet, if they were able to survive

the first few weeks of the sulfur administration. Experiments which have for their purpose the explanation of the development of this tolerance are now in progress.

Bürgi and Gordonoff (12) have recently stated that the livers of rabbits fed sulfur for a long period had a glycogen content 2 to 3 times that of normal control animals. Details of their experiments have not to our knowledge been published. In view of their findings we have determined the glycogen content of the livers of a number of our experimental animals (Table IV). It

TABLE IV.

Diet.	Glycogen content of liver, per cent.								Average.
Basal (Sherman-Mer-rill).....	1.73	6.20	3.93	1.32	0.91	0.83	1.00		2.27
Basal + 0.5 per cent sulfur.....	1.67	1.27	3.11	2.46	0.92				1.89
Basal + 0.75 per cent sulfur.....	1.51	1.80	0.87	1.25	0.81	0.59	0.52		1.05
Basal + 1 per cent sulfur.....	1.35	1.30	0.75	0.63					1.01
Basal (Osborn-Mendel).....	2.24	2.60	1.62						2.15
Basal + cystine (0.34 per cent).....	0.90	1.23	1.64	1.34					1.28
Basal + 1 per cent sulfur.....	2.16	1.39	1.89	1.90	1.27	3.93			2.09
Basal + cystine and 1 per cent sulfur.....	1.02	0.86							0.94

will be seen that although there was a considerable variation in the percentage of glycogen present, no tendency was observed for the glycogen content of the livers of the rats to which sulfur was fed to be greater than those of the control animals. The findings of Bürgi and Gordonoff do not hold for rats under the conditions of our experiments. Unfortunately, details of their investigation are not available, and we are unable to compare their experiments with our own in respect to dosage of sulfur and duration of the experiments.

SUMMARY.

Experiments are described in which flowers of sulfur in concentrations of 0.08, 0.50, 0.75, and 1.0 per cent were fed to young white rats maintained on two types of cystine-deficient basal diets (Sherman-Merrill milk powder-starch and Osborne-Mendel). No evidence was obtained to suggest that sulfur can replace cystine or in any way alter the cystine requirements for growth in the young white rat. When added to the milk powder-starch diet, sulfur was found to be definitely toxic and caused the death of fourteen out of twenty-two animals whose diet contained it to the amount of 1 per cent. After the addition of lower concentrations of sulfur to the milk powder-starch diet and the addition of 1 per cent sulfur to the Osborne-Mendel diet, marked retardation of the rate of growth was observed. The presence of amounts of cystine adequate to produce good growth did not affect the toxicity of the sulfur. The toxic effect was probably due to the formation of hydrogen sulfide in the intestine. There was no increase in the glycogen content of the livers of the rats to which sulfur had been fed, as compared with control animals.

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